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Biological Effects of Copper and Arsenic Pollution

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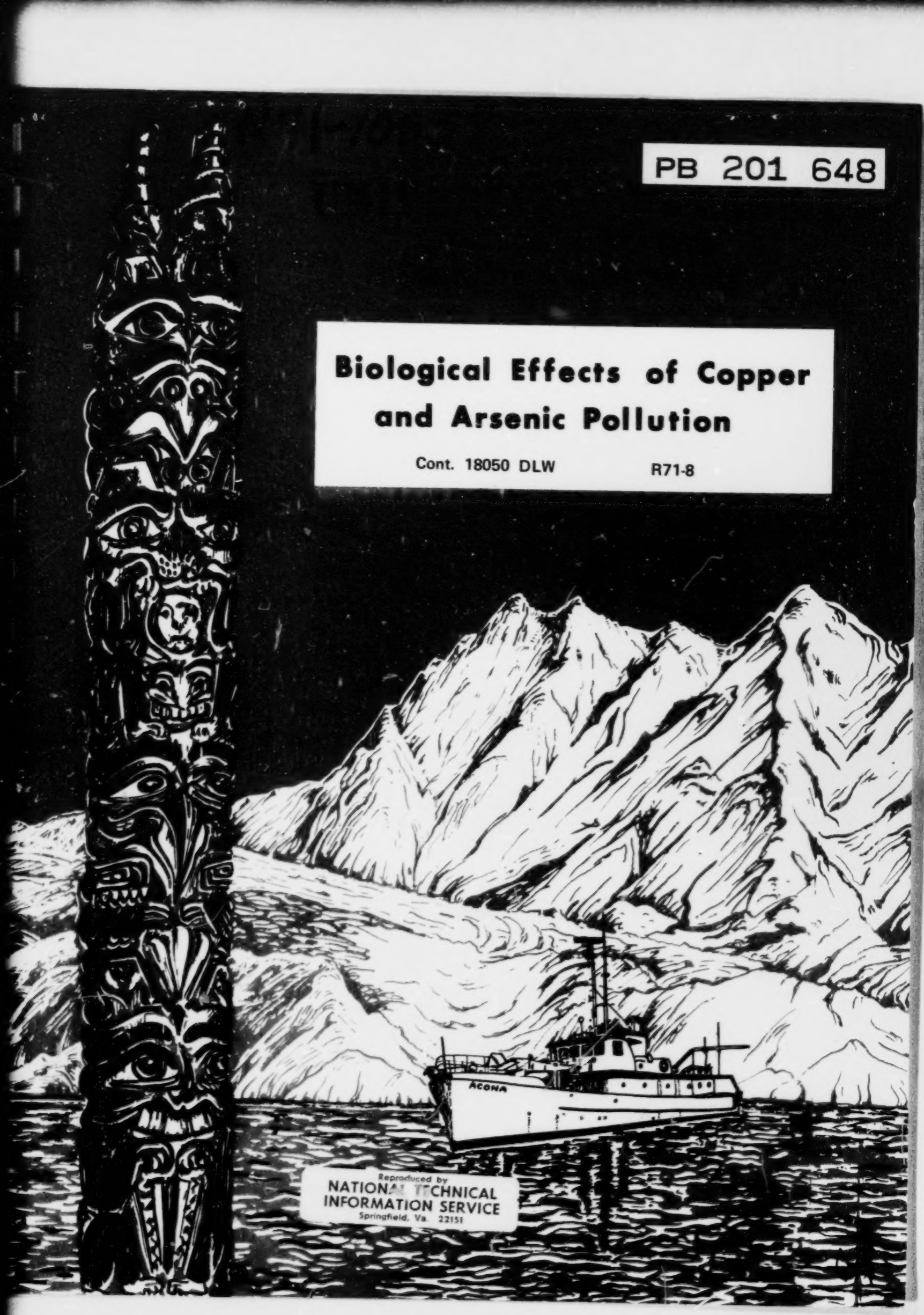

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BIOLOGICAL EFFECTS OF COPPER AND ARSENIC POLLUTION

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ABSTRACT

Inhibitory effects of copper toward marine microorganisms were studied. Phosphate was found to prevent copper inhibition. An organism capable of metabolism at low phosphate concentrations was selected. Growth and phosphate flux kinetics were described. These phosphate limited systems were found to be copper sensitive only under conditions of manganese deficiency. Toxicity occurred in this normally rather resistant yeast and in hydrocarbon oxidizing microorganisms at about 10^{-6} M. The protective influence in reagent grade phosphate was traced to its usual contamination with trace amounts of manganese.

Among a number of metabolic inhibitors found to inhibit phosphate transport was arsenate. Phosphate also competitively reduced arsenate uptake. Reduced phosphate uptake rates occurred at 10^{-6} M arsenate and high death rates in cultures otherwise sustaining normal growth rates were induced with 10^{-7} to 10^{-8} M arsenate in phosphate deficient systems. These levels are exceeded in environmental and potable water systems.

Evidence is presented consistent with the view that both arsenate and phosphate are accumulated by the same active transport system. The system is peculiar in that velocities increase as the hydrogen ion is increased, saturation of the system does not occur, and the transport temperature coefficient is very large.

Evidence suggests that both arsenate and copper are important at existing concentrations in the environment. When nutrients are dilute these anti-metabolites prevent microbial metabolism thus affecting the steady state chemistry which they control.

It is abundantly clear that toxicity levels of antimetabolites depend not only on populations and chelate concentrations but in a major way on free nutrients and trace metal levels.

INTRODUCTION

It is well known that phosphate is actively accumulated by many organisms (Blum, 1966; Goodman and Rothstein, 1968) and that the accumulation is directly effected by arsenate (Mitchell, 1964; Jung & Rothstein, 1965). However little data exists describing phosphate incorporation kinetics in the concentration range found in natural water systems. One reason for this is that organisms with the capacity to live in low phosphate natural systems have sufficient affinity and capacity to satisfy their growth requirements at less than the usual background phosphate content of chemically defined laboratory media. This paper describes a method to handle nutrient uptake kinetics and suggests that environmental levels of arsenate are such that they make this phosphate analogue an important antimetabolite in population dynamics.

That heavy metals such as copper are inhibitory is also well known (Breslow and Girotti, 1966; McBrien & Hassall, 1965; Steemann-Nielsen & Wium-Anderson, 1969; Steeman-Nielsen & Wium-Anderson, 1970; Nielsen, 1969; Cabadaj & Gdoven, 1970; and Grande, 1966). This paper further describes the strong influence the availability of divalent metabolites such as manganese has on the level at which copper becomes inhibitory. Implications of the combined influence of these antimetabolites are discussed.

METHODS

A marine yeast was selected from the collection of Dr. K. Natarajan, Institute of Marine Science, University of Alaska, on the basis of ease of growth on low phosphate medium. This pink yeast was isolated from Amukta Pass near the Aleutian Islands. It was found to be nitrate positive, lactose negative, asporogenous and otherwise similar to Rhodotorula rubra.

As with most yeasts the sodium chloride concentration did not affect maximum growth rates and was maintained low for chemical simplicity. Other constituents were maintained at near limiting values for the rather low populations employed. The constituents of the medium were (per liter): glucose, 5 mg; NaCl, 40 mg; KCl, 4 mg; NH_4SO_4, 1 mg; $MgSO_4$, 2.5 mg; $CaCl_2 \cdot 2\ H_2O$, 5 mg; $ZnSO_4 \cdot 7\ H_2O$, 70 µg; $Fe(NH_4)_2SO_4 \cdot 6\ H_2O$, 50 µg; $MnSO_4 \cdot 7\ H_2O$, 15 µg; $Co(NO_3)_2 \cdot 6\ H_2O$, 0.15 µg; vitamins B_1, B_{12} and biotin, 10^{-10} M in distilled water followed by filtration. Continuous culture was in a single phase 250 cc stirred glass reactor at 25°C as previously described (Button and Garver, 1956).

Limiting substrate concentration and growth rates in continuous culture were calculated from the usual relationships (Button, 1969) modified to analyze conditions where limiting substrate concentration S is low and approaches the quantity supplied as impurities in the feed S_b. Thus at steady state:

$$X = \gamma\ (S_a - S_b - S) \qquad (1)$$

where X is the cell population and γ the yield constant. The yield constant can be determined graphically from cell population at a range of added substrate values according to equation 1 where S and S_b are constant. The value of S_b is then given by the steady state population X_b when S_a is zero.

$$X_b = \gamma\ (S_b - S) \qquad (2)$$

A portion of added substrate was isotopically labeled so that the radioactivity ratio, R, of extracellular steady state reaction medium to total isotope concentrations gives S from the concentrations S_a and S_b:

$$S = R\ (S_a + S_b) \qquad (3)$$

Substituting S_b from equation 2 into equation 3 and solving for S:

$$S = \frac{R\,(S_a - X_b/Y)}{1 - R} \qquad (4)$$

The background substrate concentration S_b was evaluated at the beginning of each experiment according to equation 2. The portion of total substrate remaining outside the organism at steady state R was determined by a rapid separation procedure. Reactor contents were withdrawn with a syringe fitted with a two way valve which allowed immediate filtration separation of the dilute yeast population from surrounding medium. The single phase reactor was fitted with a temporary filtered air source to replace removed liquid during sampling so that the sample was not diluted with unreacted feed. The medium supply system was shut down for a sufficient period to prevent rapid replacement of the removed sample volume from appreciably disturbing the steady state.

Initial rates of uptake were measured from the slopes of organism radioactivity with respect to time, usually over a six minute period. When the pH was altered, 10^{-4} M Tris buffer was added to the medium, otherwise similar to that described. Organisms for these experiments were provided by a phosphate limited continuous culture at half maximum growth rate operated solely for this purpose. These systems were cleaned where necessary by exposing to a fresh phosphate limited culture for phosphate scavenging. No phosphate precipitates or radiochemical exchange with the glassware could be detected below pH 7.0 according to microfiltration and long term storage experiments with $^{32}PO_4$ labeled medium.

Cell mass and volumes were calculated from an integration of electronic counter and plate count population, size distribution and total volume from electronic counter data, microbalance determination of dry weight in evacuated heated chambers and checked by comparison with glucose limited continuous

cultures according to the equation $X = \gamma (S_o - S)$ (Button, 1969) where cell yields, steady state populations and the glucose distribution is known.

A gamma spectrometer was used to count arsenate, scintillation or planchett counting was used to count ^{32}P depending on the radioactivity available. Usual scintillants and precautions were used throughout.

RESULTS

During steady state growth phosphate controls growth velocity between zero and 2×10^{-8} M at pH 4.0 as shown in Fig. 1. The flux of phosphate through the cell membrane can be calculated from initial uptake experiments at concentrations greater than sufficient to achieve the maximum growth rate of the organism, and thus measure the capacity of the phosphate transport system. This flux is shown in Fig. 2. Arsenate is incorporated in a similar manner and arsenate flux is also shown as calculated from initial uptake data. Line slopes are approximately one throughout, indicating absence of a saturation mechanism. This lack of saturation indicates diffusion limitation of phosphate and arsenate transport so the system was examined for an active transport mechanism. Recent data show that phosphate and arsenate show the same affinity for the cell.

As shown in Fig. 3 plunging cells exposed to 10^{-6} M phosphate into higher concentrations loads the cells to such an extent that previously formed phosphate pools leak out. Preliminary experiments with high concentrations of phosphate suggested this from the fact that initial uptake rates were very high for the first minute, and then total radioactivity incorporated into the cell decreased.

Exchanged phosphate amounts to a minimum concentration of 5×10^{-6} M inside the cell concentrated from the 10^{-6} M external solution. The actual pool size is probably much larger, since all free phosphate would not be expected

be the case. In fact, toluene completely stopped phosphate incorporation as shown in Table I.

Table I
Phosphate Uptake Inhibition

Inhibitor	Concentration	Uptake rate M/g cells/min	Inhibition %
-	-	12.6	0
Toluene			100
NaN_3	10^{-5} M	1.6	56
IAA	10^{-5} M	8.7	31
DCCD	10^{-5} M	11.3	12
AsO_4^{-3}	10^{-7} M	4.3	36
AsO_4^{-3}*	10^{-7} M	0.4	94
AsO_4^{-3}*	10^{-8} M	5.1	20

*10 minutes pretreatment with inhibitor

Metabolic inhibitors such as sodium azide were found to be inhibitory, however, N, N-dicyclohexyldicarbodiimide (DCCD), a bound adenosine triphosphotase inhibitor, was found to be relatively ineffective. Since this system does appear to be an active transport system of sorts, ATP does not appear to be involved as suggested above and phosphoenol pyruvate is thought to drive some sugar transport systems, iodacetate was tested. It is a well known inhibitor of glyceraldehyde-3-phosphate dehydrogenase, a step preceding phosphoenol pyruvate formation. The degree of inhibition was some three times that of DCCD at 10^{-5} M and it is conceivable that phosphate transport is driven by this glycolytic intermediate.

Most striking was the inhibition of phosphate transport by arsenate, a phosphate analogue. Arsenate treated cells yielded only 6% of control activity

Fig. 1. Steady state phosphate concentration as a function of growth rate. Growth rates were calculated from continuous culture dilution rate and viability data. The maximum growth rate μ_{max} is 0.175 hr^{-1}. Phosphate concentrations are calculated from extracellular radioactivity according to the relationship

$$S = \frac{R\,(S_a - X_b/Y)}{1 - R}$$

Data spread is shown as standard deviation from the results of approximately 10,000 hours of continuous culture operation data.

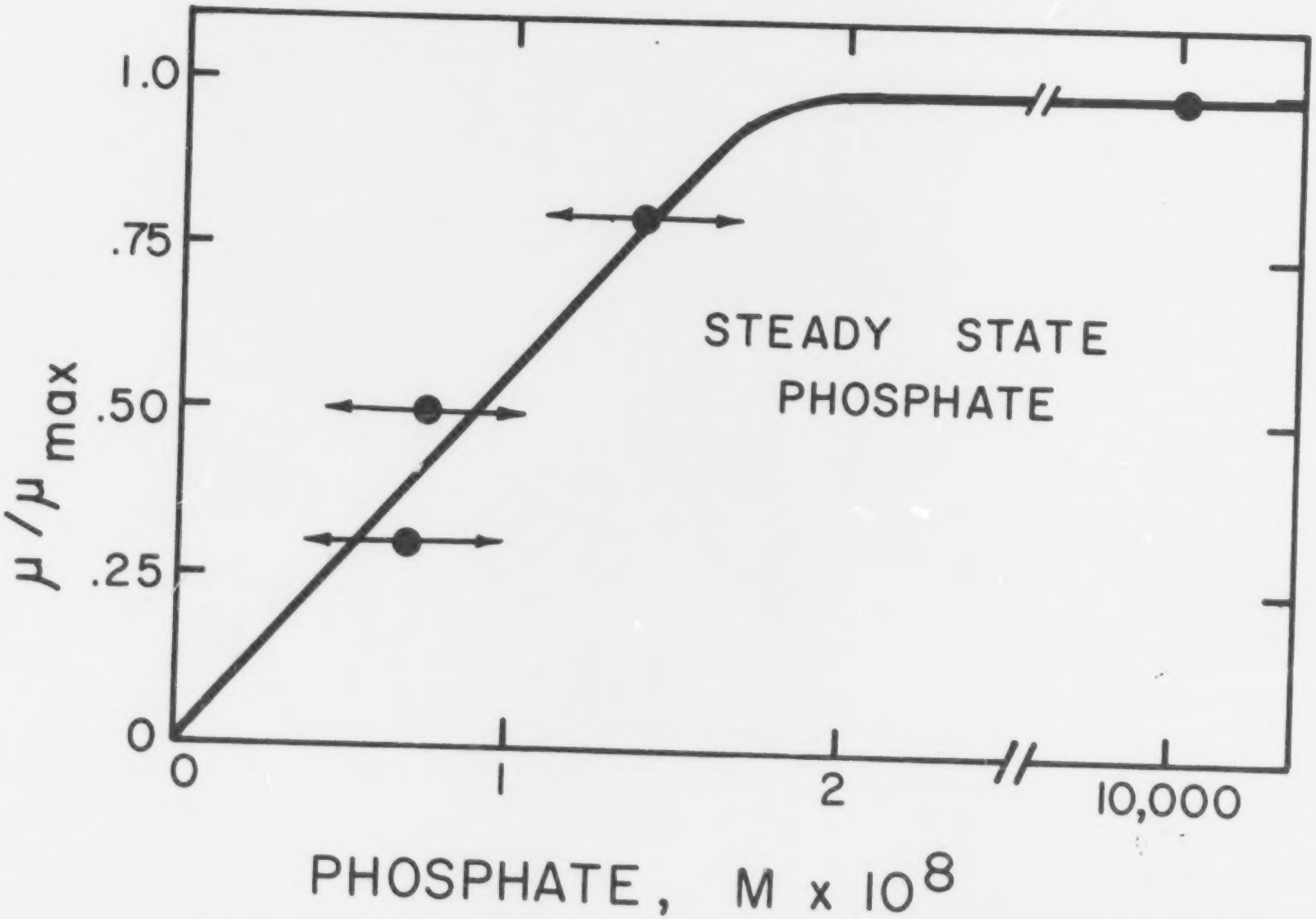

1.0
.75
.50
.25
0
μ/μ_{max}
STEADY STATE
PHOSPHATE
0
1
2
10,000
PHOSPHATE, M x 10^8

Fig. 2. Arsenate and phosphate uptake as a function of concentration. Solid symbols from initial uptake data, open symbols from steady state continuous culture data. Velocity units, moles per mg cells dry wt per minute; concentration, molar.

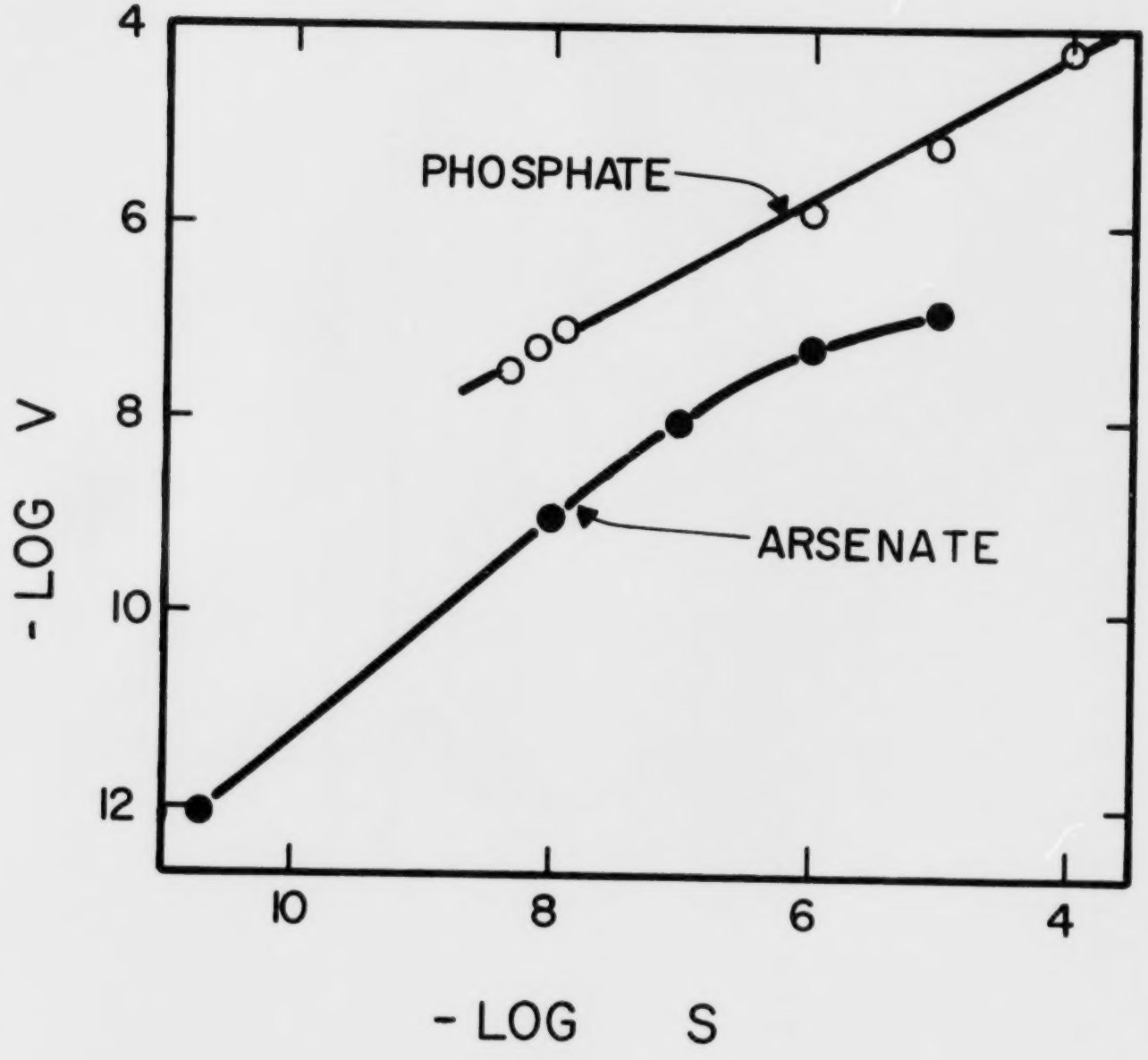
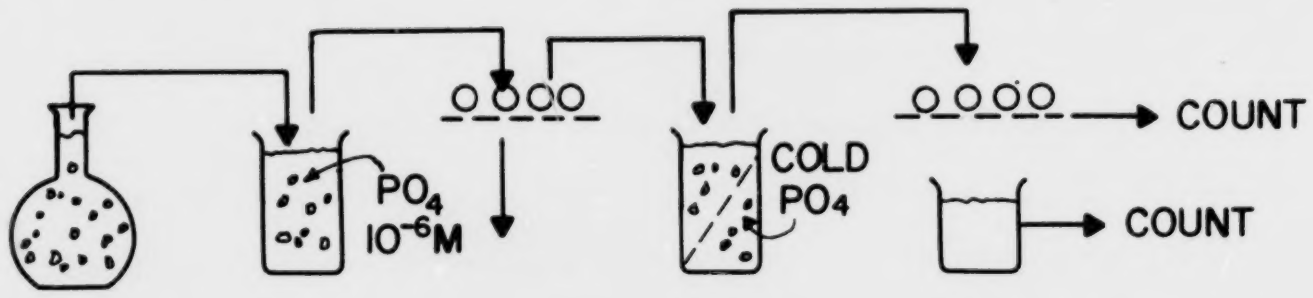

PHOSPHATE
ARSENATE
- LOG V
- LOG S
4
6
8
10
12
10
8
6
4

Fig. 3. Phosphate removed from cells by plunging cells equilibrated with 10^{-6} M phosphate into higher concentrations to allow free or pool phosphate to exchange back out. The first column shows radioactivity incorporated from labeled phosphate. The second column shows the concentration of cold phosphate and the third column shows the phosphate radioactivity remaining in the filtrate as an indication of phosphate removed from the original concentration supplied.

PHOSPHATE POOLS



UPTAKE	EXCHANGE PO_4	PO_4 REMOVED MOLES $\times 10^{12}$
1723	10^{-7} M	2.7
1469	10^{-4}	5.5
12 70	10^{-2}	5.6
333 *	10^{-7}	2.5 *
288 *	10^{-2}	2.3 *

* HEAT KILLED CONTROL

to exchange back out. Hot ethanol as well as trichloracetic acid extraction by conventional techniques shows an internal concentration of 10^{-2} M extractable ^{32}P material from 10^{-6} M phosphate.

Because these organisms are found at low temperatures and because our studies of maltose transport[a] showed similar linear uptake kinetics and operated very poorly at low temperature, we studied the effect of temperature on the phosphate transport system. Fig. 4 shows an Arrhenius plot of these data which yields an activation energy of 52,000 cal per mole. This value also is high and suggests a mechanism more complex than simple diffusion and one that requires reserve capacity for operation at low temperature.

Mitchell (1954) has suggested that only one of the four possible phosphate species might be transported. Accordingly we studied the velocity of phosphate transport with respect to hydrogen ion concentration as shown in Fig. 5. Both the initial uptake rate and the concentration at half maximum growth rate show that the resistance to phosphate incorporation is greater when the pH is raised. Most experiments were performed at pH 4 where the flux is maximum. Another reason for this choice of pH is the fact that NH_3 is the only ligand present with an appreciable binding constant, and this species virtually disappears at pH 4. This simplifies consideration of extracellular chemical complexation. Recent observations show a similar pH dependence for arsenate uptake, that is significantly higher at pH 4 than pH 7.

A number of metabolic inhibitors were tested to see what sorts of antimetabolites effected transport from a medium 10^{-6} M in phosphate. Organic solvents such as ethanol, dimethyl sulfoxide and toluene were tested because in some of our other systems showing similar kinetics, membrane disruptants have been found to increase substrate flux[a]. This however, was not found to

[a] D. K. Button, unpublished data

if the cells were presented with 10^{-7} M arsenate 10 minutes before phosphate uptake was measured as shown.

Since phosphate uptake is inhibited by both arsenate and metabolic inhibitors, part of the inhibition was probably due to reduced respiration of this strict aerobe. To see if arsenate inhibition was common with a phosphate transport step the degree of arsenate transport inhibition by phosphate was measured as shown in Fig. 6. Since arsenate transport kinetics are linear, concentrations and velocities are presented rather than their reciprocals as normally used when describing saturatable systems. The data show that arsenate transport is reduced 50% by about 10^{-6} M phosphate.

The effect of arsenate on the viability of low phosphate systems is shown in Fig. 7. Line slopes show that growth rates are slightly reduced by 10^{-8} M arsenate and that 10^{-7} M arsenate is sufficient to produce a death rate of 0.15 hr^{-1} from a control growth rate of 0.12 hr^{-1}.

Many marine isolates are inhibited by low levels of heavy metals. Table II shows some of the characteristics of hydrocarbon oxidizing organisms selected mostly from Cook Inlet, Alaska. Table III shows their sensitivity to copper. All showed reduced growth rates and most were killed by the addition of 10^{-6} M copper as shown including R. rubra used in all of the preceding experiments. However early experiments showed that high phosphate concentrations prevent copper inhibition of growth rates or viability at any level and generated the need to understand phosphate incorporation kinetics described in the foregoing. This understanding allowed experimentation with steady state continuous culture systems at low but not limiting levels of phosphate. Fig. 8 shows a pair of glucose limited continuous cultures population data. One set has a moderate (10^{-5} M) level of phosphate but trace metals are at only the level provided by reagent grade chemical contamination. The second set are with a full complement of trace metals but with added

Fig. 4. Initial rate of phosphate uptake measured over a six minute period with respect to temperature from a 10^{-6} M medium according to the Arrhenius equation. Velocity is expressed as moles phosphate per gram cells dry weight per minute. Temperature shown is degrees centigrade. Temperature plotted is the reciprocal of the absolute temperature.

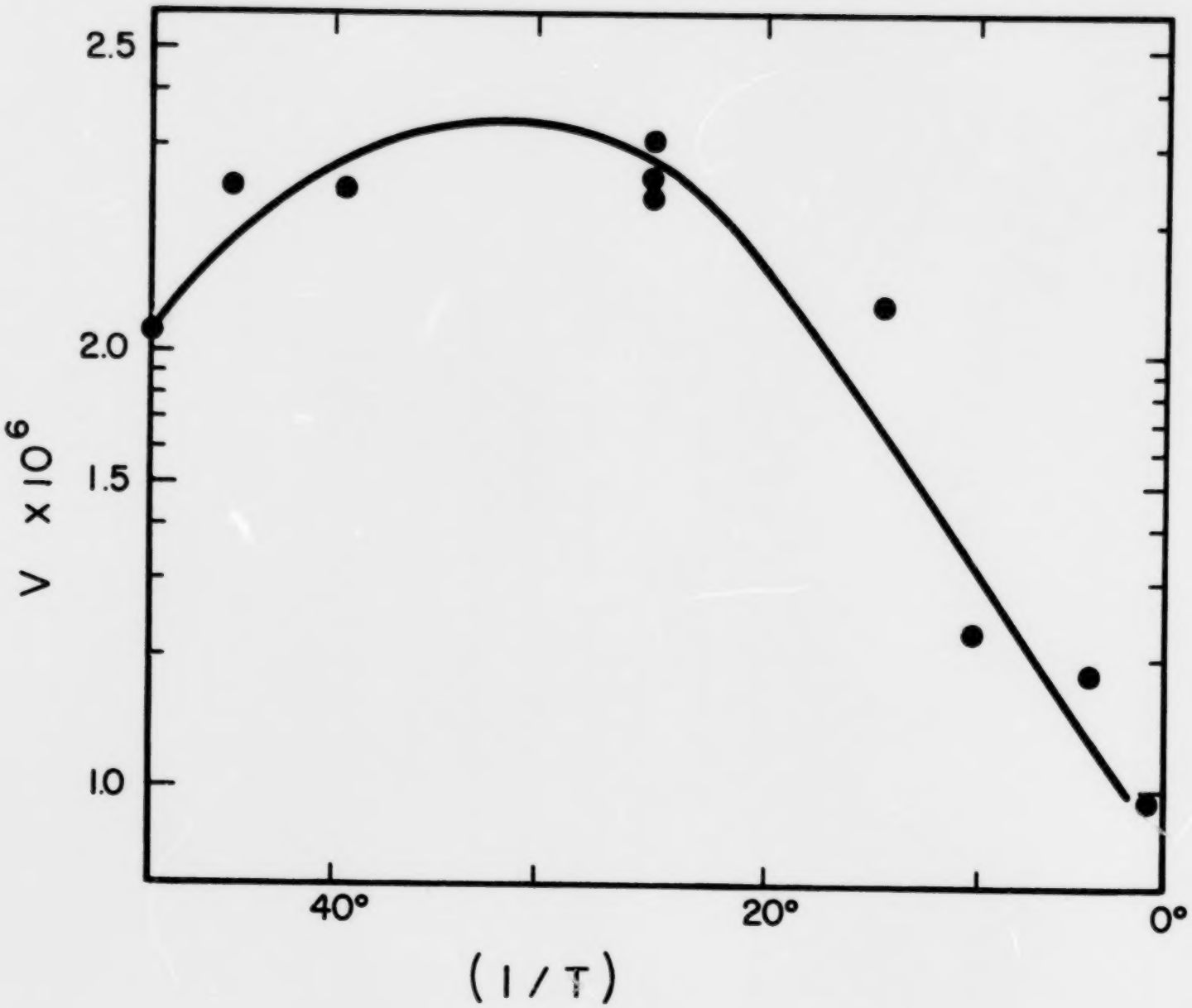

2.5
2.0
1.5
1.0
V x 10^6
40°
20°
0°
(1/T)

Fig. 5. Effect of hydrogen ion concentration on phosphate flux. Open symbols, steady state concentration of phosphate in a continuous culture system diluted at 0.09 hr^{-1}. Closed symbols, initial uptake rate from a 10^{-6} M phosphate solution. Concentrations are expressed as total extracellular phosphate.

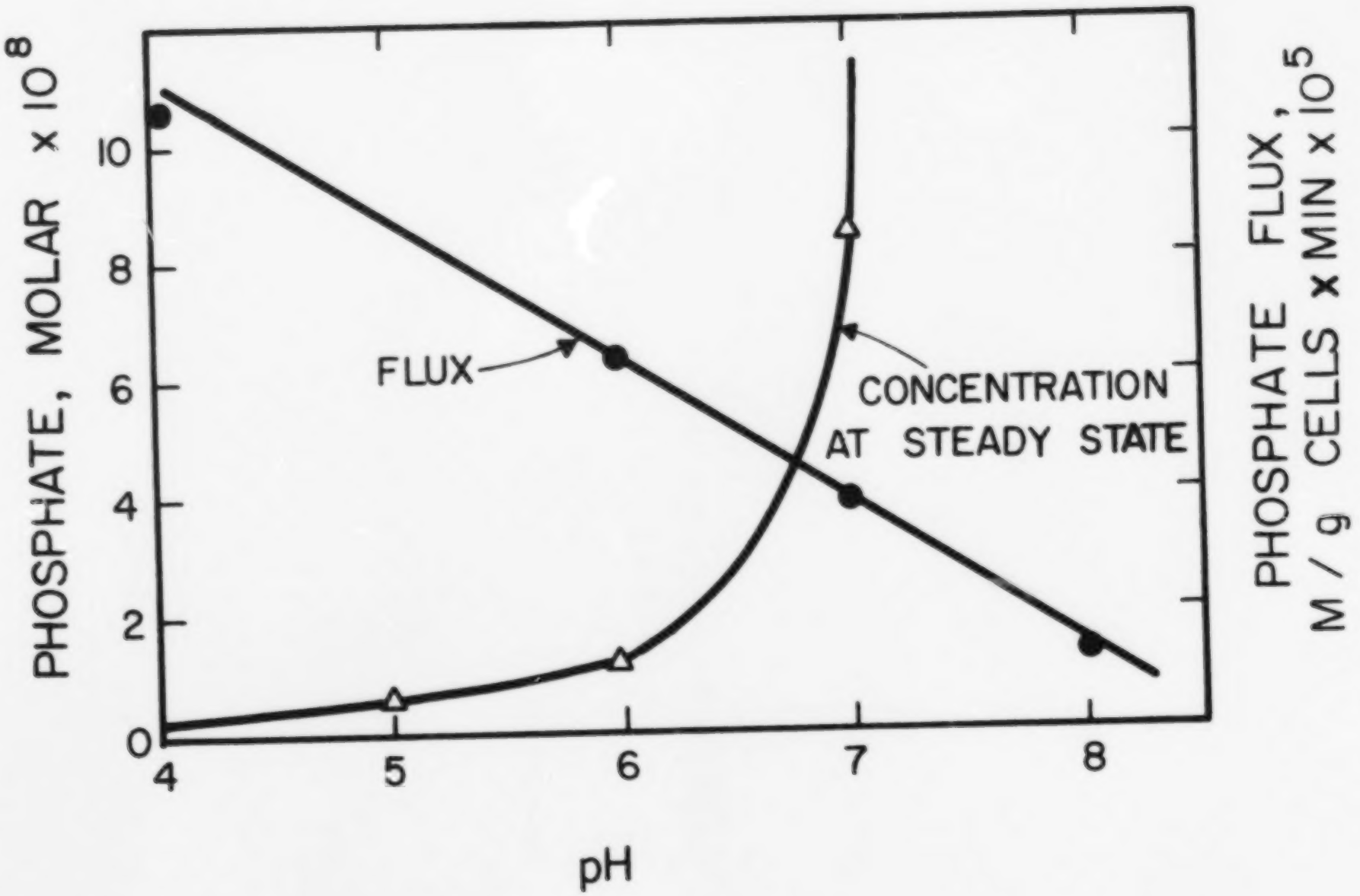

PHOSPHATE, MOLAR x 10^8
10
8
6
4
2
0
4
5
6
7
8
pH
FLUX
CONCENTRATION
AT STEADY STATE
PHOSPHATE FLUX,
M / g CELLS x MIN x 10^5

Fig. 6. Initial rate of arsenate transport with respect to arsenate concentration with three concentrations of phosphate.

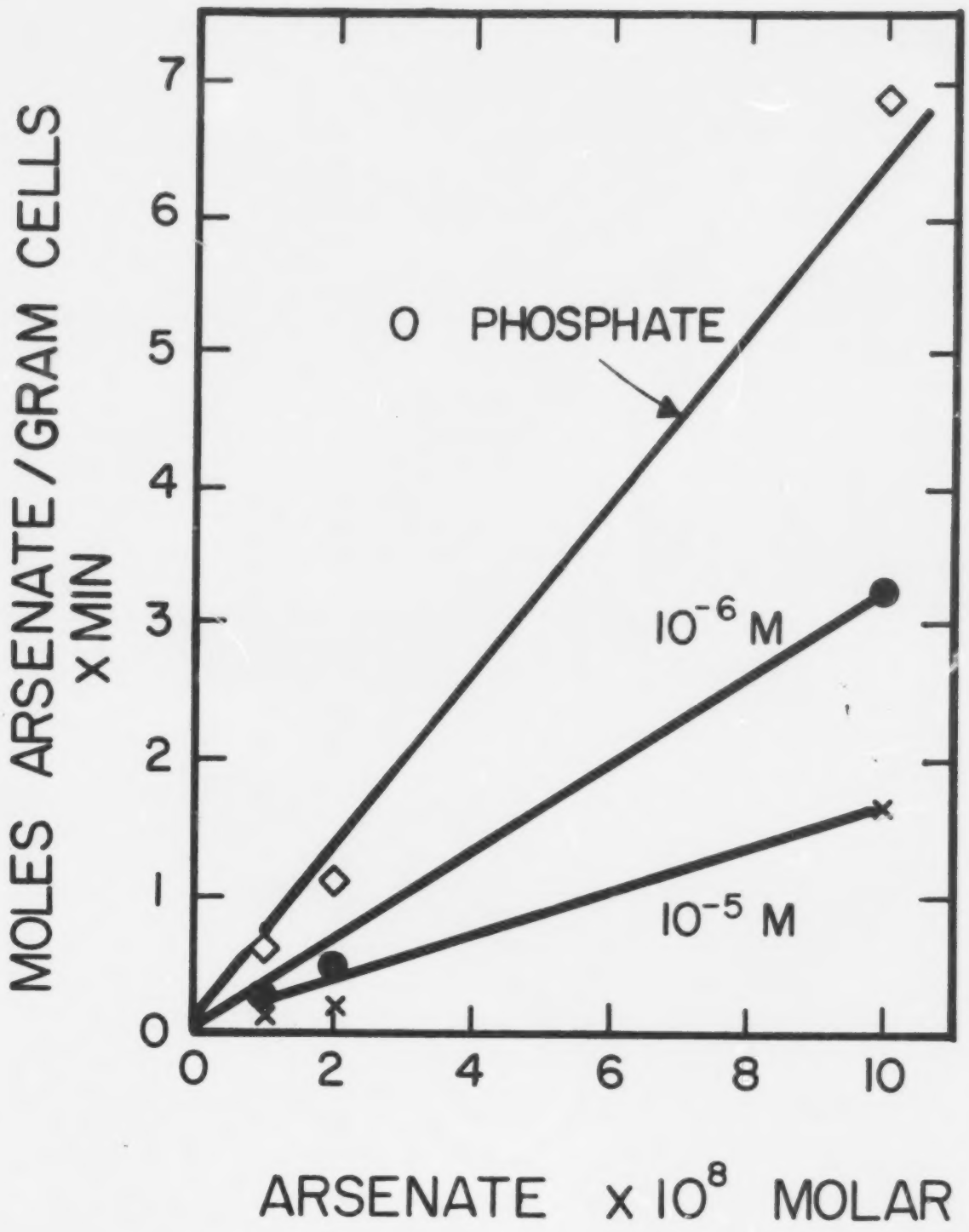

MOLES ARSENATE / GRAM CELLS
X MIN
0
1
2
3
4
5
6
7
0 PHOSPHATE
10^{-6} M
10^{-5} M
0
2
4
6
8
10
ARSENATE X 10^{8} MOLAR

Fig. 7. Growth rates in low phosphate solution at various levels of arsenate. Inoculation was from a phosphate limited continuous culture and populations measured by plate counting.

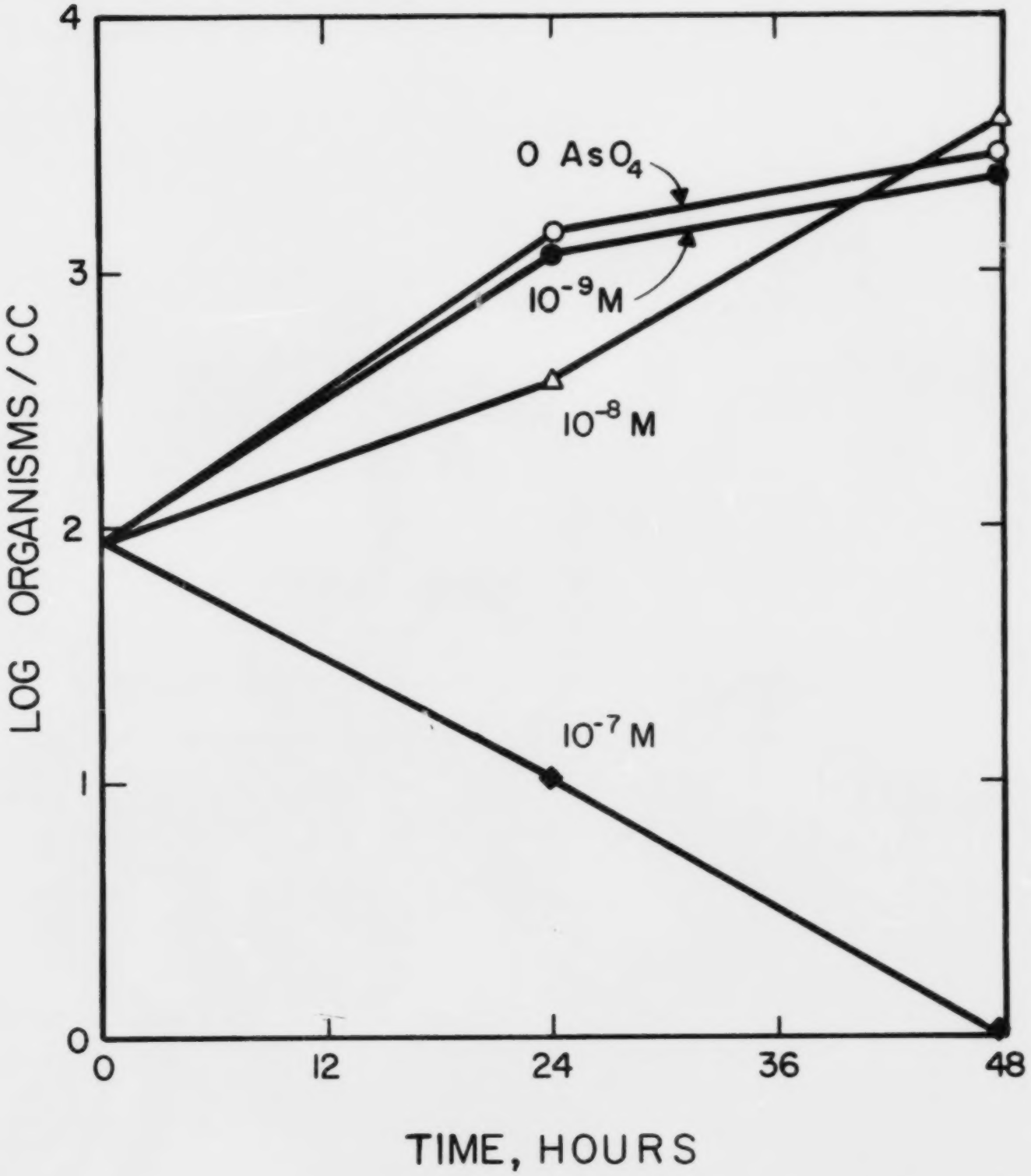

LOG ORGANISMS / CC
4
3
2
1
0
0
12
24
36
48
TIME, HOURS
0 AsO4
10-9 M
10-8 M
10-7 M

Fig. 8. Effect of heavy metals on the growth rate of R. rubra. Data are steady or transient state populations in continuous culture of two runs. Circles are electronic counter counts, triangles are plate counts. Both runs are glucose limited at 5×10^{-6} M. Medium used in the upper run contains 10^{-5} M phosphate but no added zinc, manganese, cobalt or molybdenum. The lower run medium contains these added trace elements but phosphate is reduced to 3×10^{-6} M.

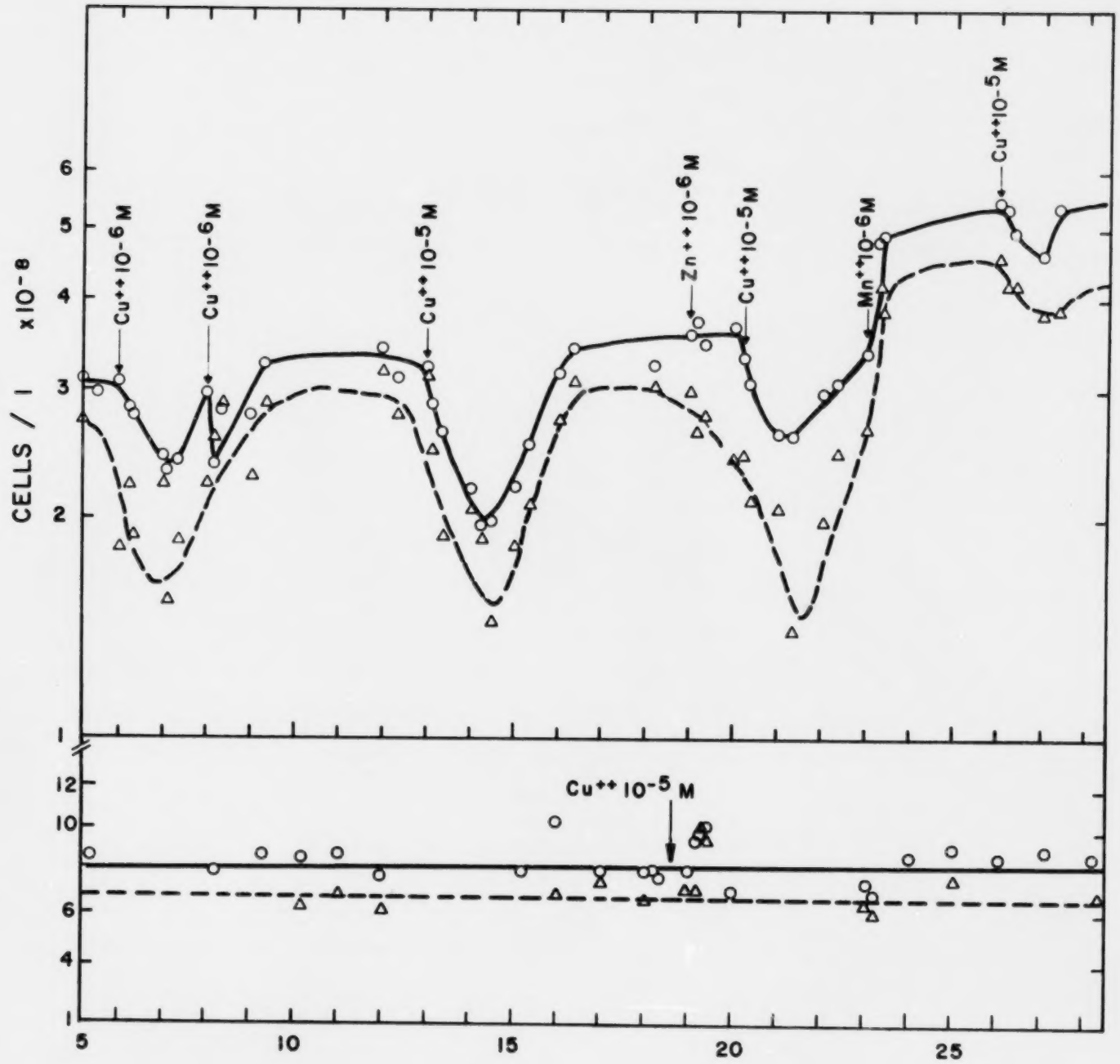

CELLS / 1 x10-8
Cu++10-6M
Cu++10-6M
Cu++10-5M
Zn++10-6M
Cu++10-5M
Mn++10-6M
Cu++10-5M
Cu++10-5 M
TIME, DAYS

Table II. Hydrocarbon oxidizing organism characteristics.

Culture	Gram	Dimensions µM	Shape	Upper temp. limit	μ_{max} broth hr^{-1}	μ_{max} kerosene hr^{-1}	Ea K/cal/mole	Emulsification*	Type**
54	+	0.6 x 1.0	rod	30	0.23	-	-	2	Mycobacterium
72	+	0.5 x 2.0	rod	30	0.35	0.20	16.0	1	Nocardia
80	+	1.0 x 1.2	coccoid	37	0.35	0.087	-	1	Arthrobacter
114	+	0.6 x 3.0	rod	30	0.35	0.020	16.0	2	Nocardia
179	+	0.6 x 3.0	rod	25	0.35	-	15.2	2	Nocardia
181	-	0.5	cocci	25	0.15	-	-	0.5	Micrococcus
197	+	0.5 x 20	branched filaments	-	-	-	-	-	Streptomyces
198	+	0.6 x 3	rod	25	0.35	-	14.5	0.5	Mycobacterium

*Genus most closely resembling isolate.

**Grams crude oil emulsified per gram dry weight of organism.

Table III

Culture	Added Cu^{++} 0	10^{-6} M	10^{-5} M	10^{-4} M
54	5×10^{4}*	0	0	0
72	3×10^{4}	0	0	0
80	1×10^{6}	0	0	0
114	1×10^{6}	3×10^{5}	0	1×10^{4}
179	5×10^{4}	1×10^{3}	1×10^{3}	1×10^{2}
181	70	0	0	0
197	70	-	5×10^{3}	0
198	70	0	0	0
R. ruba	370	20	0	0

*Cultures were grown in nutrient broth, harvested and suspended in mineral mannose medium pH 7.2 except R. rubra which was suspended in mineral salts glucose medium pH 4.0. Columns above report the population in broth culture 24 hr after inoculation with various quantities of added copper shown.

phosphate at only 3×10^{-6} M so that added copper at 10^{-5} M is in a greater than 1:1 ratio with remaining phosphate after population growth. It is clear that the trace metal deficient steady state is repeatedly perturbed by added copper showing no accumulated resistance and that manganese and to a lesser extent zinc reduce the inhibitory effects of copper. The low phosphate high trace metal system remains unperturbed under the same conditions of perturbation as shown in the upper data set. Atomic absorption analysis of our stock phosphate revealed a manganese peak of sufficient size to provide an alternate manganese source and thus provide protection from copper inhibition in high phosphate systems.

DISCUSSION

The isotope dilution bioassay technique presents a useful method to handle nutrient incorporation kinetics of a common metabolite incorporated by a high affinity system. The data spread for phosphate uptake appears large but the absolute concentrations involved are extremely small, less than 1% of that found in most marine systems. Although it appears that phosphate uptake rates extrapolated to a finite concentration at zero concentration, extensive data collection (10,000 hours of continuous culture operation) did not substantiate this point or reduce the data spread. Initial uptake data were obtained to evaluate the capacity of the phosphate transport system and are the basis for describing the phosphate incorporation system in growing systems as nonsaturatable. The mechanism of phosphate uptake remains obscure. However the linear concentration velocity relationship has appeared before and it may be a rather general type of incorporation kinetics. Active transport is clearly implicated in that internal free phosphate concentrations are greater than external concentrations. Metabolic inhibitors such as iodoacetate and sodium azide also support the view that active transport is

involved, possibly driven by phosphoenol pyruvate hydrolysis since DCCD appears ineffective.

The fact that arsenate inhibits phosphate transport, phosphate inhibits arsenate transport, and that the uptake kinetics are similar suggest that this is another example of a transport system that cannot distinguish between the two ions.

If the mechanism involves diffusion limitation of a transfer process across a membrane one might expect a low temperature coefficient for transport and that membrane solubilizing agents might enhance the transport rate. We found neither to be the case and none of the usual transport models appear attractive to explain the results presented.

The environmental implications of these findings are interesting. Table IV shows some typical arsenate levels occurring in potable water and marine

Table IV

Environmental Arsenic[a]	
Soil	10,000 ppb[b]
Hot springs	8,500
Lakes	2 - 50
Rivers	0.2 - 25
Desert ground water	8 - 27
Sea water	1 - 5
Rain water	0.02 - 14

[a] Weoephl, K. H., Handbook of Geochemistry, New York, 1969, p. 33-I-1.

[b] 1 ppb is equivalent to 1.4×10^{-8} M

systems. Dr. Michael Pilson[a] reports marine arsenic concentrations of between 10^{-8} and 10^{7} M with the concentration in some areas exceeding that of phosphate. Since arsenic is particularly effective in low phosphate systems it seems clear that existing arsenic levels are sufficient to effect the metabolism rate of at least part of a diversified population in low phosphate natural systems. It seems likely that low levels of arsenate have had a role in contributing to some of our experimental irreproducibility. For example, sodium chloride tolerance experiments using this organism probably more accurately reflected the arsenate level in the reagent grade sodium chloride used than the salt tolerance of the marine yeast.

The survey of various hydrocarbon oxidizing marine organisms at hand showed major copper toxicity at 10^{-6} M. However most of the foregoing was an effort to track down the rather variable sensitivity a rather resistant marine yeast exhibited toward copper. The final continuous culture shows that copper inhibition is related in a major way to trace metal nutrition. This is not particularly surprising when one realizes that the prosthetic groups of many enzymes can be replaced by heavy metals under certain conditions. It does however mean that many organisms sensitive to heavy metals are probably even more sensitive than reported when in natural systems which are trace metal and particularly manganese deficient. Effects of this copper - manganese interaction await careful quantitation.

It seems likely that growth rates of organisms in the natural environment are reduced by antimetabolites such as arsenate and heavy metals and at existing concentrations so that blooms only occur when nutrient build-up has been sufficiently great to provide a positive driving force to autocatalytic microbial reproduction sufficient to overcome the negative influence of anti-

[a]Personal communication, Dr. Michael Pilson, Univ. of R. I.

metabolites discussed. It is interesting that this "bloom or bust" type behavior of aquatic populations is probably one that maximizes the standing crop from available nutrients. The alternative of a stable slow growing steady state population in the ocean for example would distribute available nutrients so widely that major amounts of energy would be diverted to endogenous metabolism in the smaller primary forms and to collection mechanisms in the upper trophic levels. The expected result would be a lower yield of fish for example. However some environmental arsenate levels may greatly exceed threshold toxicity levels and seriously disturb the normal metabolic processes. Reinforcement of these effects would be expected by additional antimetabolites such as heavy metals.

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APPENDIX I

DATES AND TITLES OF CONTINUOUS CULTURE RUNS AND EXPERIMENTS

CONTINUOUS CULTURE DATA

Run	Dates	Experiments (Rh-rubra)
76	5/15/68 - 5/26/68	Cu^{++} inhibition @ low constant phosphate concentration.
77	6/6/68 - 7/8/68	Added Cu^{++}, $P6Cl_2$, $HgCl_2$, $ZnSo_4$ to observe effect on phosphate limited culture.
79	8/1/68 - 8/23/68	Effect of copper on glucose limited steady state continuous culture.
80	9/4/68 - 10/7/68	Glucose limited steady state culture w/o copper.
81	10/15/68 - 12/18/68	Added $MnSo_4$, varying amounts of substrate - glucose and copper to reactor @ 50% maximum growth rate.
86	11/10/69 - 1/17/69	Phosphate limited steady state continuous culture.
87	3/13/69 - 2/24/69	Phosphate limited steady state continuous culture with P^{32}.
88	3/13/69 - 4/4/69	Phosphate limited steady state continuous culture with P^{32}.
90	4/9/69 - 5/12/69	Phosphate limited steady state continuous culture with respect to pH and determination of S.
91	5/19/69 - 6/19/69	Determination of S @ phosphate limited steady state continuous culture @ $\bar{r}0.3$, $\bar{r}0.5$ + $\bar{r}0.8$.
92	6/24/69 - 7/8/69	Phosphate limited steady state culture determination of S @ different pH values.
94	7/8/69 - 7/23/69	Increasing amounts of phosphate added to phosphate free steady state continuous culture.
95	8/6/69 - 9/9/69	Phosphate limited steady state continuous culture samples taken for S @ $\bar{r}0.3$, $\bar{r}0.5$ + $\bar{r}0.8$.

Run	Dates	Experiments (Rh-rubra)
96	9/23/69 - 10/6/69	Low phosphate steady state continuous culture.
96R	10/6/69 - 10/23/69	Phosphate limited steady state continuous culture with respect to pH.
96Rx2	10/23/69 - 12/8/69	Various sampling method for S in steady state continuous culture phosphate limited @ $\bar{r}0.3$.
100	12/4/69 - 1/8/70	Phosphate limited steady state continuous culture and determination of S @ $\bar{r}0.3$, $\bar{r}0.5$ + $\bar{r}0.8$ using new quick sampling method.
101	1/16/70 - 2/12/70	Phosphate limited steady state continuous culture $\bar{c}$ determination for S @ $\bar{r}0.8$.
105	2/16/70 - 2/26/70	Low phosphate steady state continuous culture $\bar{c}$ determination for S @ $\bar{r}0.8$.
106	3/2/70 - 3/14/70	Phosphate limited steady state continuous culture $\bar{c}$ determination for S @ $\bar{r}0.8$.
107	3/2/70 - 4/11/70	Effect of Ni^{++} on K_s for phosphate.
110	4/15/70 - 5/8/70	Determination of S with Ni^{++}, @ $\bar{r}0.5$.
113	6/8/70 - 6/13/70	Determination of S with copper, @ $\bar{r}0.3$.
114	6/22/70 - 7/9/70	Phosphate limited continuous culture @ steady state $\bar{c}$ sample taken for S @ $\bar{r}0.5$.
120	8/18/70 - 9/14/70	Determination of $\bar{S}$ with $\bar{C}^{14}$ and copper samples taken @ $\bar{r}0.5$ + $\bar{r}0.8$.
127	10/7/70 - 11/11/70	With a phosphate limited continuous culture contamination of effect of Cu^{++} $MnSO_4$ + $ZnSO_4$.

D. K. Button - FROM NOTEBOOKS

Ref.	Date	Expt.
DKB B4 P13	1/5/68	Inoculation + isolation of a low phosphate requiring organism.
DKB B4 P14	1/11/68	Set up culture in yeast extract, isolated 3 colonies and transferred to low glucose media and allowed to grow through one log phase.
DKB B4 P31	1/20/68	A check for amounts of phosphate in media salts.
DKB B6 P4		Copper inhibition test for hydrocarbon microbacterium isolate.
DKB B6 P6		Copper inhibition of continuous culture @ steady state @ pH7.
DKB B6 P9	6/9/70	Copper inhibition of continuous culture using ^{64}Cu.
DKB B6 P10	6/10/70	The use of low pH copper to prevent clumping copper @ 3×10^{-6} M.
DKB B6	7/2/70	N.N'-Dicyclohexylcarbodiimide inhibition of phosphate transport.
DKB B6 P14	7/2/70	Initial uptake of phosphate c̄ glucose 5 mg/l.
DKB B6 P15	7/17/70	A viability check with phosphate + copper.
DKB B6 P16	7/20/70	Initial uptake from continuous run, using phosphate @ concentrations of 1×10^{-4} M → 1×10^{-8} M.
DKB B6 P17	7/22/70	Effect of pH on phosphate uptake.
DKB B6 P18	7/24/70	Phosphate uptake of C. utilis + C. Albidus.
DKB B6 P19	7/28/70	Phosphate concentration gradient in Rh rubra.
DKB B5 P32	11/11/69	Phosphate uptake in synthetic media.
DKB B5 P56	1/20/70	Expt. for Rh rubra mutant.

Ref.	Date	Expt.
DKB B5 P66	2/25/70	Low phosphate transport of mutants.
DKB B5 P67	2/27/70	Continuation of phosphate mutant expt.
DKB B5 P73	5/4/70	Preparation of phosphate transportless mutants.

INITIAL UPTAKES (HEAVY METALS)

Ref.	Dates	Expts.
SSD BK2 P9	8/12/70	Copper inhibition of phosphate uptake pilot run.
SSD BK2 P10	8/13/70	Copper inhibition of phosphate uptake pilot run II.
SSD BK2 P14/15	8/17/70	Copper inhibition of phosphate uptake run III.
SSD BK2 P69-73	12/2/70 → 12/7/70	As-0_4 @ 10^{-6} M, 10^{-5} M, 10^{-7} M, 10^{-8} M + 10^{-9} M pilot expts.
SSD BK3 P32	12/21/70	As-73 c AsO_4 initial uptake.
SSD BK3 P33	12/29/70	Phosphate inhibition of AsO_4 uptake.
SSD BK3 P35	12/30/70	Comparison of As-0_4 + phosphate flux under identical conditions.
SSD BK3 P38	11/7/71	AS-73 pool concentration expt.
SSD BK3 P45	1/14/71	AS-0_4 toxicity to Rh rubra.
SSD BK4 P33	3/22/71	Effect of pH on AS-0_4 uptake.

Notes: * = day, * = expts. were repeated

INITIAL UPTAKES (OTHER)

Ref.	Dates	Expts.
SSD BK2 P12	8/14/70	Phosphate concentration gradient a range of dilutions from 1 x 10^{-6} M phosphate → 10^{-7} M phosphate.
SSD BK2 P13	8/17/70	Effect of temperature on phosphate uptake, 5°C, 15°C, 25°C.
SSD BK2 P16	8/18/70	Effect of temperature on phosphate uptake, 5°C, 15°C, 25°C.
SSD BK2 P17	8/20/70	Phosphate concentration gradient a range of dilutions from 1 x 10^{-4} M phosphate → 5 x 10^{-6} M phosphate.
SSD BK2 P18	8/21/70	Effect of temperature on phosphate uptake, 10°C, 20°C + 25°C.
SSD BK2 P19	8/21/70	Phosphate inside Rh rubra cells (pools).
SSD BK2 P27	8/31/70	Glucose requirement of phosphate transport.
SSD BK2 P35	9/14/70	Effect of temperature on phosphate uptake, 1°C.
SSD BK2 P37	9/21/70	Initial uptake regarding pH $\bar{c}$ phosphate limited cells.
SSD BK2 P39	9/29/70	Effect of sodium + potassium.
SSD BK2 P44	10/2/70	Effect of pH on phosphate limited cells (final expt.).
SSD BK2 P47	10/7/70	Repeat of salt concentration effect using sodium and potassium $\bar{c}$ phosphate limited cells.
SSD BK2 P53	10/22/70	Effect of pH on phosphate limited cells $\bar{c}$ tris buffer.

INHIBITORS

SSD BK2 P11	8/13/70	N.N'-dicyclohexyldicarbodiimide (DCCD) effect on phosphate uptake.

Ref.	Dates	Expts.
SSD BK2 P56	11/11/70	Iodoacetic acids, effect on phosphate uptake.
SSD BK2 P58	11/13/70	Sodium azide's effect on phosphate uptake.
SSD BK2 P74	12/7/70	Sodium dichromate effect on phosphate uptake.
SSD BK2 P75	12/11/70	Effect of high temperature on phosphate uptake, 25°C, 30°C + 35°C.
SSD BK3 P21	12/16/70	Effect of high temperature on phosphate uptake, 25°C, 40°C + 50°C.
SSD BK3 P36	1/4/71	Effect of low temperature on phosphate uptake, 25°C, 40°C, 50°C, 1°C.
SSD BK4 P7	2/16/71	Phosphate inhibition of AS-0_4 uptake, 25°C, 40°C, 50°C, °C.
SSD BK4 P8	2/17/71	Phosphate uptake c̄ sodium chloride.
SSD BK4 P12	2/22/71	Saline inhibition check for viability.
SSD BK4 P14	2/24/71	Phosphate inhibition @ low temperature, 5°C, 15°C + 25°C.
SSD BK4 P25	3/8/71	Effect of EtOH on phosphate transport.
SSD BK4 P26	3/9/71	Repeat of EtOH on phosphate transport and including effect of dimethylsulfoxide (DMSO) 20%.

APPENDIX II

EFFECT OF CLAY ON THE AVAILABILITY OF DILUTE ORGANIC NUTRIENTS TO STEADY-STATE HETEROTROPHIC POPULATION

SOME FACTORS INFLUENCING KINETIC CONSTANTS FOR MICROBIAL GROWTH IN DILUTE SOLUTION

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EFFECT OF CLAY ON THE AVAILABILITY OF DILUTE ORGANIC NUTRIENTS TO STEADY-STATE HETEROTROPHIC POPULATIONS

By D. K. BUTTON

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EFFECT OF CLAY ON THE AVAILABILITY OF DILUTE ORGANIC NUTRIENTS TO STEADY-STATE HETEROTROPHIC POPULATIONS[1]

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ABSTRACT

The effect of clays on the availability of small organic molecules to microorganisms was determined. Recovered suspended sedimentary material and Bentonite was tested for its ability to compete with yeast and bacteria for thiamine or glucose in continuous culture systems at low organism concentrations. The added clays do not render the organics tested unavailable to microorganisms or remove them from solution to a detectable or significant extent. The addition of clays can, however, effect a perturbation to the steady system, possibly by altering the inorganic chemistry of the dissolved phase.

INTRODUCTION

Chemical analyses of certain systems have been interpreted to show that clays and sediments are effective in adsorbing organic compounds from solution. These clay-organic associations include starch (Lynch, Wright, and Cotnoir 1956), proteins and amino acids (Sieskind and Wey 1959), and carbohydrates (Bader, Hood, and Smith 1960). The amount of adsorption determines the effect of clays on the dissolved organic chemistry of sediment-laden water systems. Suspended sediment loads normally run from 0.5 to 100 mg/liter in the glacier-fed bays and inlets of southeast Alaska and even higher in streams draining areas of intensive mining or other earth-moving activities.

The extent of the clay-organic association was further investigated by measuring the influence of clay particles on the nutrient adsorption kinetics of some heterotrophic cultures. This was done by subjecting steady-state low population nutrient-limited cultures to perturbation by injected clay.

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[2] I am indebted to Emma Dieter who provided technical assistance.

THEORY

In a nutrient-controlled continuous culture system, the limiting nutrient governs the steady-state cell population (Monod 1950; Herbert, Elsworth, and Telling 1956; Button and Garver 1966) and is divided between the growing organisms and extracellular solution according to the equation:

$$X = Y(S_0 - S), \qquad (1)$$

where S_0 is the initial limiting nutrient concentration and Y the yield constant, grams of organisms produced per gram of limiting substrate used, $(S_0 - S)$. In continuous culture at 100% viability the average growth rate is numerically equal to the dilution rate and is set by the concentration of S at a particular temperature. This rate increases as a monotonic increasing function of substrate concentration frequently approximated by the Michaelis-Menten equation:

$$\mu = \frac{\mu_{max} S}{K_s + S}. \qquad (2)$$

Thus one can determine changes in available substrate concentration, S, by observing changes in the steady-state concentration of organisms, X, at a particular rate. If one chooses a value of S_0 near the Michaelis constant for growth, K_s, and sets the dilution rate (which is equal to the growth rate at 100% viability and perfect population dispersal) at a large fraction

of the maximum growth rate, μ_{max}, small changes in available limiting substrate are reflected by large changes in the cell population. For example, K_s for *Escherichia coli* is 4 mg glucose/liter (Monod 1950), about the same as for most heterotrophs near their upper temperature limit under usual laboratory conditions. If one operates at a dilution or growth rate of 0.5 μ_{max}, where $S = K_s$, and sets S_0 at 5 mg/liter, the resulting population at $Y = 0.5$ will be 0.5 mg/liter, according to equation (1). Under these conditions a 10% reduction in available substrate would reduce the steady-state bacterial population to half its original value.

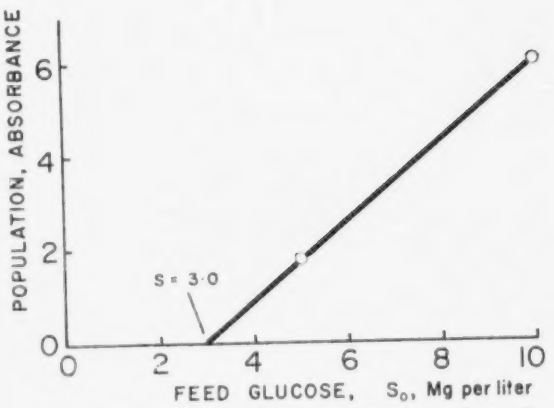

FIG. 1. Plot of absorption at 625 mμ of steady-state *Cryptococcus albidus* populations at a dilution rate of 0.5 max or 0.132 hr^{-1} according to equation (1).

CLAY PREPARATION

Initial nutrient adsorption experiments were run with sediments collected from routine hydrographic stations in southeast Alaska. Sufficient seawater was filtered to obtain a few hundred mg of sediment. The sediment was weighed, resuspended, and autoclaved 15 min at 121C. This sterile suspension was either introduced into the feed chamber (a 20-liter carboy) or the culture vessel (a 250-ml boiling flask) of the continuous culture apparatus, depending on the experiment. Sterile conditions were maintained by injecting the suspensions with a syringe through rubber covered ports. Standard clay was prepared by grinding Montmorillonite No. 27 (Bentonite: Belle Fourche, S. Dak.; Ward's Natural Science Establishment, Inc., Rochester, N.Y.) in a porcelain mortar and pestle. This was fractionated by sedimentation and a cut of approximately 1.2×10^{-13}-g particles was obtained with 90% of the particles between 1.5 and 2.5 μ in diameter. These particles remained independent from one another and from the microorganisms; this was clear in phase contrast examination of the suspensions.

CULTURE METHODS

Nutrient adsorption by clay was measured in a single-phase continuous culture apparatus of the type described by Button and Garver (1966). *Cryptococcus albidus*, a thiamine-requiring yeast, average volume 65 μ^3, was used in the thiamine-limited and *p*H 4 glucose-limited experiments. This microorganism behaves well in continuous culture without tending to stick and will grow on a simple chemically-defined medium. The strain used was isolated from the marine system (Natarajan and Dugdale 1966) and is carried in the Institute of Marine Science culture collection. The constituents of the medium were (per liter): $NaH_2PO_4 \cdot H_2O$, 2 g; $(NH_4)_2SO_4$, 4 mg; KCl, 40 mg; $MgSO_4$, 25 mg; $CaCl_2 \cdot 2H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 0.7 mg; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 25 μg; $MnSO_4 \cdot 7H_2O$, 1.5 μg; $CoSO_4 \cdot 7H_2O$, 1.5 μg; and MoO_3, 0.5 μg. Glucose and thiamine when not limiting were added to 100 mg/liter and 10^{-9} M respectively and the *p*H was adjusted to the desired value with NaOH. This medium was designed to be near minimal in as many aspects as possible to simplify consideration of chemical interactions.

For experiments at neutral *p*H, *E. coli* B-4 was selected because of its high maximum growth rate and *p*H optimum near that of seawater. This strain did adhere to the reactor walls, but it was satisfactory in short-term continuous culture experiments. The medium was the same as listed above except that $Na_2HPO_4 \cdot H_2O$ was added to 20 g/liter and *p*H was adjusted to 7.0.

A third organism was selected for use in experiments involving low buffer capacity and high *p*H—the only one of those tested that would tolerate these conditions. This organism, a pink yeast tentatively identified as belonging in the genus *Rhodotorula*, was isolated from Amukta Pass near the Aleutian Islands by K. Natarajan. It possessed the requisite characteristics of high growth rate and stable behavior in continuous culture without adhering to glass surfaces. Culture conditions were 25C, *p*H 6.5 in the medium described above except that $Na_2HPO_4 \cdot H_2O$ was reduced to 3 mg/liter. This is referred to as the "low phosphate" medium.

COUNTING AND SIZING

Populations were counted on plates prepared from the liquid culture medium plus 1.5% agar. Where possible an electronic counter (Coulter Counter Model B, Hialeah, Fla.) was used for counting and sizing the organisms and the clay. Agreement between methods was normally within statistical error when more than 1,000 colonies were counted. Yeast cells could be counted in the presence of clay with the counter owing to their greater average volume; however, all populations reported in the presence of clay were determined from plate counts. Populations for the K_s measurement were determined from optical density in a 5-cm cell at 625 mμ.

RESULTS

A thiamine-limited continuous culture of *C. albidus* was maintained at steady state at half maximum growth rate of 0.132 hr^{-1} corresponding to a doubling time of 5.25 hr at 25C. The feed contained 2×10^{-12} M thiamine, and the Michaelis constant (extracellular concentration at $\mu = 0.5_{\mu max}$) for this nutrient was 4.7×10^{-13} M (unpublished data). This resulted in a reactor population of 2.0×10^7 cells/liter. Clay recovered from Glacier Bay, Alaska was then injected into the feed to a level of 10 mg/liter of medium. This remained in suspension owing to the agitation caused by air bubbles from the sparger used to saturate the feed medium with air. The time allowed for equilibration was 48 hr, during which 6.3 volumes of feed flushed through the 500-ml reactor. The final population in the reactor after equilibration was 2.4×10^7 cells/liter, essentially the same as before adding the clay. A fall in the cell population had been expected due to the addition of the clay-thiamine (vitamin B_1) term in the equilibrium:

$$\begin{aligned} &\text{Clay} - B_1 \\ &\quad + \text{Cells} \rightleftharpoons \text{Clay} + B_1 \\ &\qquad\qquad + \text{Cells} \rightleftharpoons \text{Clay} + \text{Cells} - B_1 . \end{aligned} \quad (3)$$

However, since the above evidence indicated a low binding constant for the clay-thiamine association, the binding ability of clay was further tested with the carbon source—glucose. Experimental conditions to test the affinity of clay for glucose were the same as above except that thiamine was added to 10^{-9} M, and glucose was added to 6 mg/liter. Under these conditions, the Michaelis constant for glucose is 3.0 mg/liter as shown in the plot of equation (1) in Fig. 1 according to the method of Button and Garver (1966). The reactor population before and after adding 140 mg of Bentonite of 10 liters of feed is shown in Fig. 2. The upward portion of the curve indicates that the experiment was terminated before a true steady state was reached. However, the glucose removed by the clay cannot have exceeded 0.7 mg/liter and appears to approach zero.

Since *p*H is a factor in the chemistry and charge of the clay surface (Sieskind and Wey 1959), the apparent lack of adsorption was tested again at neutrality. For this purpose *E. coli* was set up in continuous culture at *p*H 7, with a feed glucose concentration of 6 mg/liter and at a dilution or growth rate of 0.35 hr^{-1}. The yeast used in the experiments above was not used here because its glucose transport system apparently fails above *p*H 5. The total lack of effect of Bentonite particles injected directly into the reactor on the steady-state cell population is shown in Fig. 3. As the clay particle concentration changed from zero to 10^{12} particles/liter

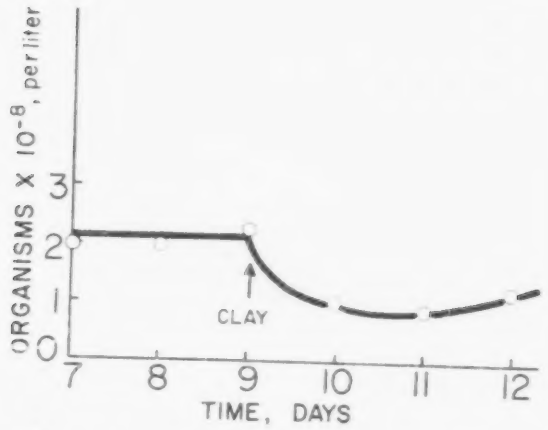

FIG. 2. Steady-state population of *Cryptococcus albidus*, glucose limited at 6 mg/liter growing at 0.132 hr^{-1} before and after adjusting the level of clay in the feed to 14 mg/liter.

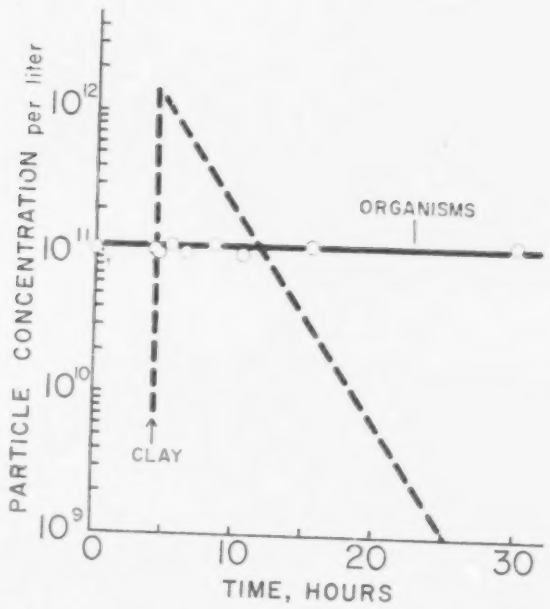

FIG. 3. Steady state of population of *Escherichia coli* growing at 0.35 hr^{-1} before and after the introduction of 10^{12} clay particles/liter into the reactor.

and then exponentially towards zero the bacterial population remained unchanged. A monomolecular glucose layer on the clay would have reduced the dissolved glucose level 4 mg/liter to 2 mg/liter and caused immediate and complete washout of the culture.

Phosphate was the only nutrient in large excess in these experiments. To rule out the possibility of phosphate protecting the clay from associations with the organic compounds, the low phosphate medium was used with the pink yeast described above. This yeast possessed characteristics allowing low population culture techniques without buffers or chelates, and it behaved very well in continuous culture. It has a thiamine requirement that is satisfied by something less than the 9×10^4 molecules/organism required by *C. albidus* at half maximum growth rate.

The chemical species having the highest binding capacity for cations in the low phosphate medium is NH_3 which is present at only 9×10^{-7} M at *p*H 6.5. The steady-state population in the presence of the 2×10^{-5} M phosphate, or low phosphate medium, is shown in Fig. 4. The initial addition of Bentonite to the reactor at 4.5 days caused a temporary 80% decrease in the cell population. The level added was 20 mg of clay/liter. However, subsequent additions of 10 and 100 mg/liter of clay to the feed had no effect on the population. Under these conditions, there was some coalescing of the clay in the feed vessel so that the reactor clay concentration was somewhat lower than the feed concentration. In Fig. 5, the effect of 100 mg of clay added to one of duplicate batch cultures 9 hr after inoculation is shown. The cultures were limited to about 2×10^7 organisms/liter by the 3 mg/liter of glucose present initially. The added clay had no effect on the final cell population; the two cultures behaved as duplicates within the normal variability of batch cultures.

DISCUSSION

The vitamin thiamine is a cation in solution with a positive charge residing at the nitrogen (3-position) of the thiazolium ring under the conditions reported here. Since clays have been reported to possess ion exchange capacity for cations (Hendricks 1941) and to have surface affinity for neutral molecules (Bader et al. 1960), it is surprising that no binding of glucose or thiamine was detected in any of the experiments reported here.

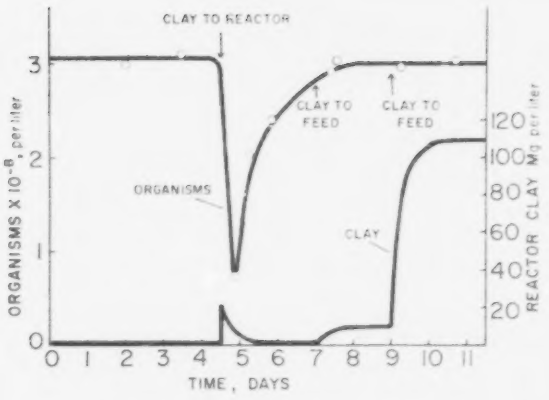

FIG. 4. Steady-state yeast population, glucose limited in unbuffered medium, as clay was added to the reactor and to the feed.

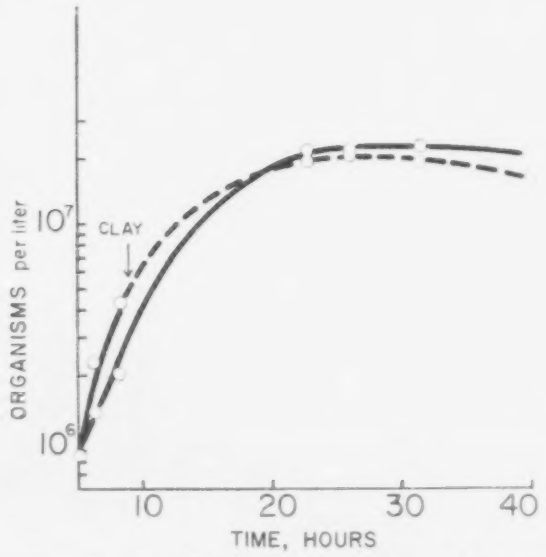

FIG. 5. Duplicate batch cultures of yeast, glucose limited at low phosphate concentrations. Arrow indicates the addition of clay to a level of 100 mg liter to one of the cultures.

Temporary reduction of a yeast population owing to the addition of clay in the low phosphate experiments was detected. Since the populations returned to the initial steady-state level and did not again decrease when subsequent and even larger clay additions were made to the feed, the effect could not have been due to nutrient removal. The temporary reduction in cell population was not surprising since biological membranes are particularly sensitive to perturbation when not stabilized with a buffer such as phosphate or a chelate ligand. The failure of subsequent additions of clay to cause similar perturbations can be explained by the normal capacity of microorganisms to respond to new situations after a suitable induction period (Horecker, Thomas, and Monod 1960). Thus it seems likely that a chemical change, such as a rise in concentrations of free heavy metals, caused the temporary response shown in Figs. 2 and 4. Recent experiments (unpublished) have shown similar temporary inhibition by injected copper at 10^{-7} M in a medium of low buffer capacity. Stotzky and Rem (1967) reported the absence of an inhibitory effect in experiments using a clay culture medium; this was probably due to the protection afforded by the yeast extract component of their basal medium.

Under the various experimental conditions described here, the data show that the equilibrium constant between free organics and those bound to suspended sedimentary material lies far in the direction of the dissolved state. The experimental conditions included a range of *p*H, neutral and charged substrates, media of high and low buffer capacity, and different types of clay preparations and concentrations. Thus, it seems unlikely that in natural systems the level of suspended organisms using small organic molecules is materially influenced by the level of suspended sedimentary material. The data do show evidence that clays cause temporary reduction of the growth rates of the organisms used, which is probably due to the solution of some component.

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SOME FACTORS INFLUENCING KINETIC CONSTANTS FOR MICROBIAL GROWTH IN DILUTE SOLUTION

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A growing microorganism reproducing in dilute aqueous environments such as our natural water systems is faced with the problems of recognizing and concentrating desired nutrients, preventing the concomitant entry of chemical species not required and retaining desired components within itself. This is successfully accomplished by simple diffusion accompanied by a set of transport systems, each capable of recognizing a required species and carrying it across the cell wall to a site of chemical modification so that the nutrient cannot move back by the same path. The nutrient molecules a species of microorganism can recognize are genetic parameters and some transport systems can be produced or omitted in response to the chemical environment. These systems are sufficiently enzyme-like to behave in a similar kinetic fashion and can be described with maximum velocities, apparent Michaelis constants and inhibition constants. These kinetic constants are a measure of effective collision frequency at the site of the growth rate limiting step as compared with the growth rate when that step is saturated.

The rate limiting step for a microorganism grown in dilute solution can therefore be nutrient diffusion through the aqueous environment to the cell surface, transport of recognized nutrients through the cell surface into the organism or a subsequent slow step in the conversion of the nutrient into useful biochemical forms. The kinetics of each step except the first can be affected by the external environment of the organism. The following observations describe how growth velocity can be affected by nutrient concentration, mixing, temperature, pH and heavy metal concentration.

Growth velocity is normally an increasing function of limiting substrate concentrations. This can be hyperbolic, linear or logistic depending on the rate limiting mechanism. The bottom curve of Fig. 1 shows how the growth velocity of a yeast cell *Cryptococcus albidus* varies with the vitamin thiamine at the cell surface. The curve is computed from the Michaelis-Menton equation, the variation in cell yield with growth rate, the variation in organism size with growth rate and the apparent Michaelis constant for growth experimentally determined and reported elsewhere (Button, 1969). The upper curve is based on a computation of the concentration of thiamine required in the bulk of the medium to supply thiamine at the cell surface at the required rate assuming no mixing and Flickian diffusion. A convenient equation presented by Borkowski and Johnson (1967) for this purpose is presented below. As one can see the effective concentration at the cell surface is about half that in the bulk of the medium in an unmixed system. Normal growth rates of yeast, bacteria and algae are indicated to provide a reference scale. These data would

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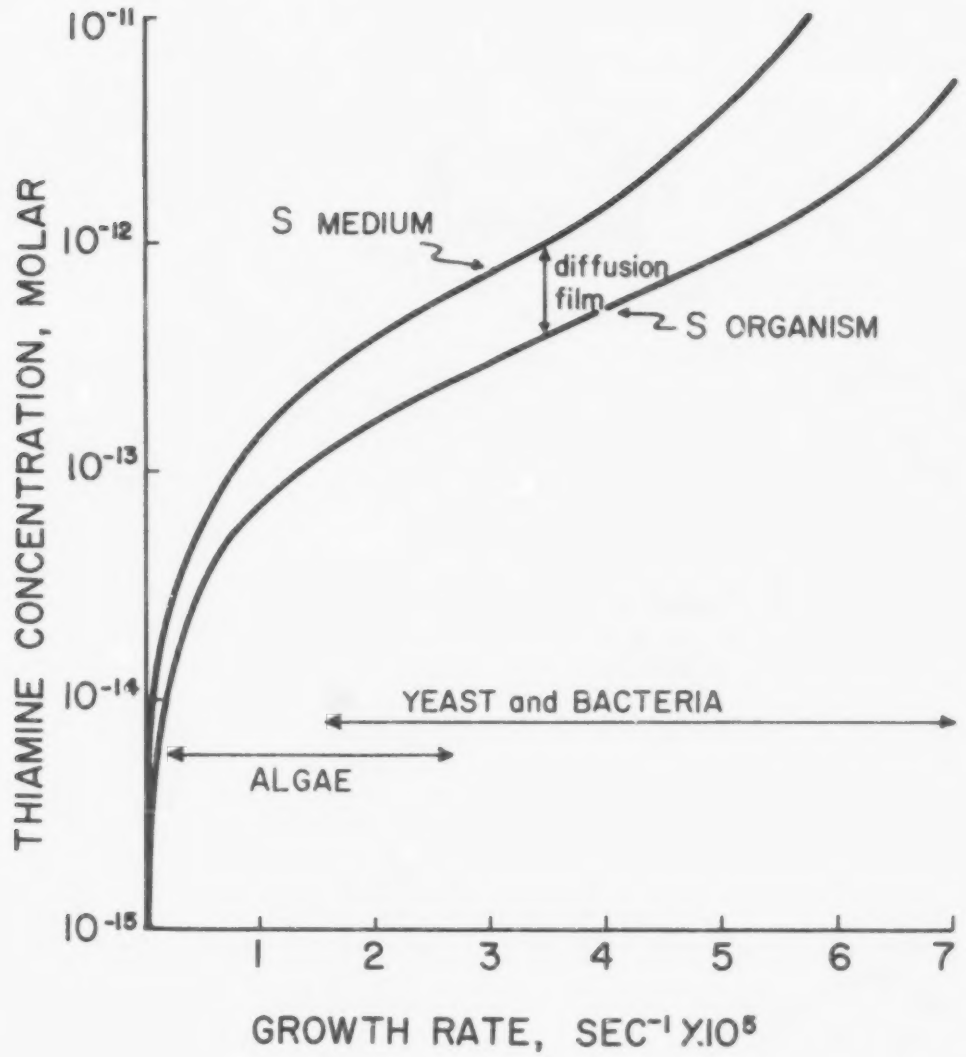

Fig. 1. The lower curve represents the thiamine concentration at the surface of the organism, *Cryptococcus albidus*, computed from the Michaelis Menton equation, K_s 4.4 x 10^{-13} M, and the changes in cell volume and yield with growth rate. The upper curve represents the thickness of the diffusion film in a non-mixed solution computed from equation 1.

indicate that in this case of vitamin limited growth, diffusion to the cell surface is the growth rate limiting step. If that is the case, the thiamine utilization rate is a function of mixing. We found this to be the case experimentally (Button, 1969).

Fig. 2 shows how the stagnant film thickness would vary with growth rate if a concentration of nutrient equivalent to its apparent Michaelis constant were provided in the nutrient medium. Curves shown were obtained as described by Borkowski and Johnson (1967). Using the experimentally determined Michaelis constant for thiamine limited growth of 4×10^{-13} M (center line) as the concentration at the cell surface, the film thickness at usual growth rates of $5 \times 10^{5}\ sec^{-1}$ is about one micron, which is within the normal range of stagnant film thicknesses. The upper and lower lines represent how the film thickness would vary if the apparent Michaelis constant were either ten times larger or ten times smaller than its actual value. The divergence suggests the existence of a rational relationship between the Michaelis constant for a substrate and the amount of substrate required by a microorganism. This was first noticed in tabulations of published Michaelis constant data. The resulting relationship is about what one would expect on the basis of molecular collision frequency in the *Salmonella typhimurium* system at published concentrations of sulfate binding enzyme (Dreyfuss, 1964) and assumed values of sulfate flux. The same relationship can be obtained from the *C. albidus*-thiamine system by substituting the film thickness at half the maximum growth rate of 5.4×10^{-4} cm, obtained from Fig. 2, into the general equation for relating diffusion limited nutrient concentration drops presented by Borkowski and Johnson (1967).

$$Cf - Cs = \frac{0.1\, r^2\, \mu\, (1-1/x)}{D\lambda} \qquad (1)$$

where Cf and Cs are concentrations of nutrient in the bulk of the medium and at the cell surface; μ is the growth rate; x is the distance from the cell center to the outside of the film in units of r, the cell radius; D is the diffusivity of the nutrient in the medium; and λ is the yield of cell mass from nutrient provided. If the experimental values of these constants for the *C. albidus*-thiamine system are substituted into equation (1) the following relationship is obtained:

$$\lambda = \frac{1}{7 \times 10^{7}\, K_s} \qquad (2)$$

A logarithmic plot of this equation is shown in Fig. 3. Notice that the experimental values of Wright and Hobbie (1965) and Borkowski and Johnson (1967) for acetate and oxygen, respectively, fall near this line, although the yield constants are a factor of 10^6 lower than the thiamine data used to formulate the relationship.

Growth velocities of microorganisms when the rate limiting step is substrate saturated follow the Arrhenius equation as shown in Fig. 4. Pena (1955) suggested a similar relationship to temperature. Our values of K_s for

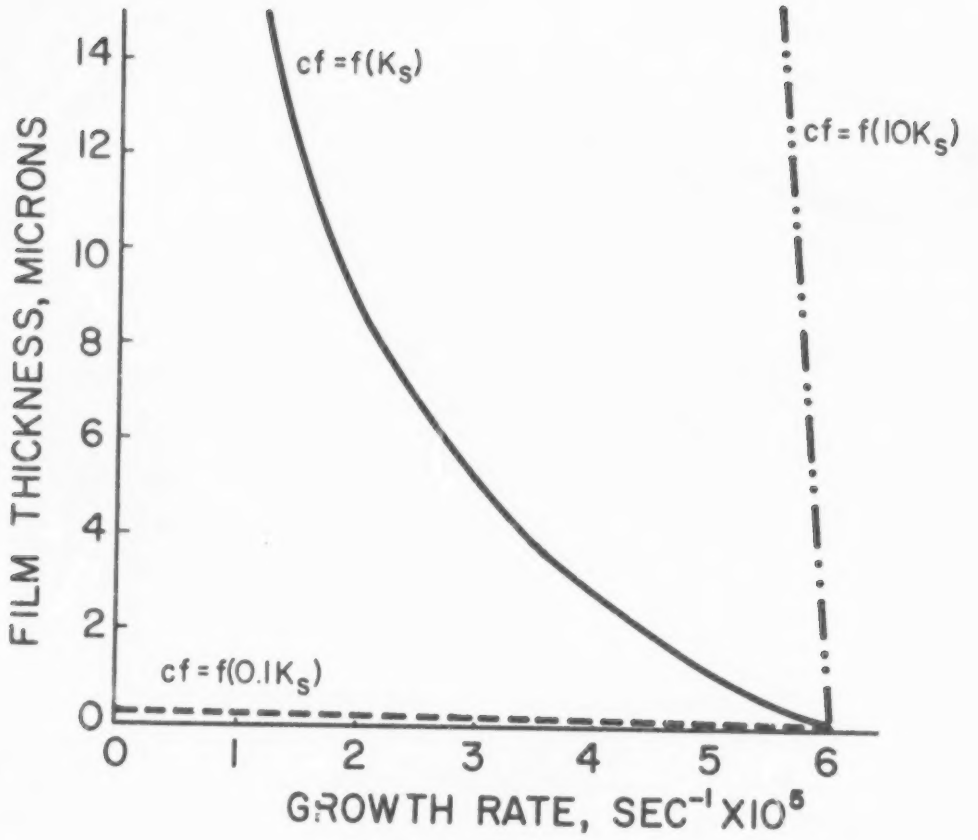

Fig. 2. The center curve (solid line) represents the maximum diffusion film thickness that will allow the indicated growth rates with the concentration in the bulk of the unmixed medium equivalent to the Michaelis constant for thiamine in the thiamine requiring *Cryptococcus albidus* system. The darkened lines show the same maximum film thicknesses when the bulk medium is raised or lowered by a factor of ten.

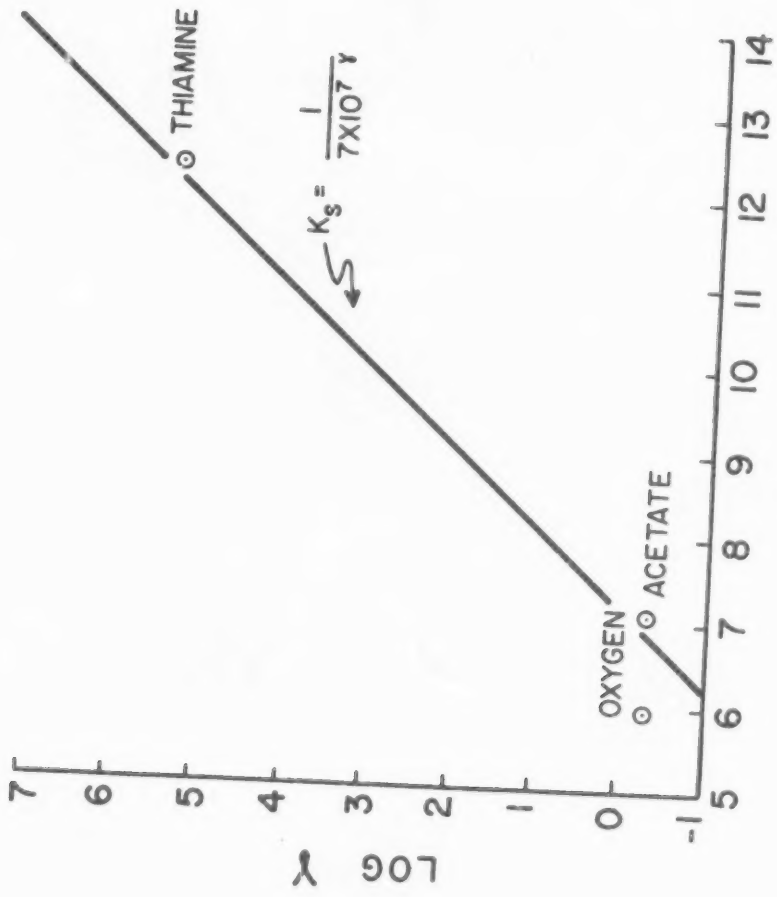

Fig. 3. Fit of experimental K_s data to equation 5. Points to the left of the line represent departure from diffusion limited systems.

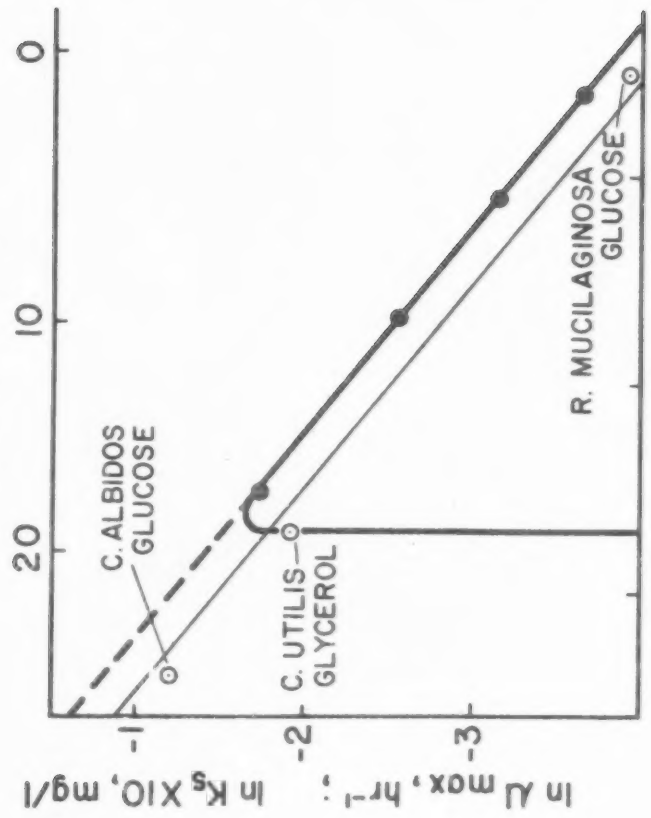

Fig. 4. Arrhenius plot of V_{max} for *R. mucilaginosa* (solid circles), and K_s vs. temperature (open circles).

glucose at 1.6 C are shown along with the value of K_s for glucose at 25 C with *C. albidus* and Pena's value for glycerol using *C. utilis* at 15 C. The values for these yeast K_s data increase with temperature in the same way as the maximum velocity of growth. Thus if

$$\mu_{max} = A\, e^{-E_a/RT} \tag{3}$$

then

$$K_s = A'\, e^{-E_a/RT} \tag{4}$$

and from (1)

$$A'\, e^{-E_a/RT} = \frac{1}{7 \times 10^7 \lambda} \tag{5}$$

Where growth rate is limited at the cell surface by nutrient transport the system can be likened to substrate competition for passage through a matrix of doors. These doors at the cell surface are chemical in nature and subject to closing (chemical modification) by inhibitory components of the medium.

Table 1 shows the maximum growth rate of *C. utilis* after extended growth at steady state in continuous culture. Population was regulated by flow rate at a low value so that the population remained at μ_{max}. The rates observed were different from the corresponding batch growth rates and much slower after the first three days. Notice that the rate responds to glycerol concentration in a different way at pH 4 than at pH 6, indicating a Michaelis constant of a higher order of magnitude at pH 6. This difference between batch and continuous culture data also occurred with *C. albidus* and *Rhodotorula glutinis* and is under current consideration. The data show that long term substrate limited growth at low population can respond to concentration in a different way than short term substrate uptake.

Fig. 5 shows the response of *R. glutinis*, a marine pink yeast, to copper. The yeast was selected because it grows well in continuous culture with no added chelates or buffers and only substrate quantities of phosphate (Button, 1969). Under these conditions the rate of growth was sharply reduced, about 70%, by the addition of copper to a final concentration of 10^{-6} M. The copper content of the continuous culture reactor decayed at the rate shown and the population approached its original value of 1.5×10^8 cells/liter. Succeeding additions of heavy metals had progressively less effect on the growth rate. However, the steady state population was reduced to about 1×10^8 cells/liter as shown. This indicates a higher heavy metal tolerance at the expense of a less efficient transport system which lowers the total standing crop at a given level of nutrients.

In summary, mixing, temperature, nutrient concentration and inhibitor concentration all affect the efficiency and rate at which microbial processes occur and effects can be described in a rational manner.

Table 1. The maximum growth rate of *C. utilis* after extended growth at steady state in continuous culture.

Glycerol (mg/liter)	pH	μ_{max} (hr^{-1})
5	4.0	0.425
1000	4.0	0.425
5	6.2	0.024
1000	6.2	0.144

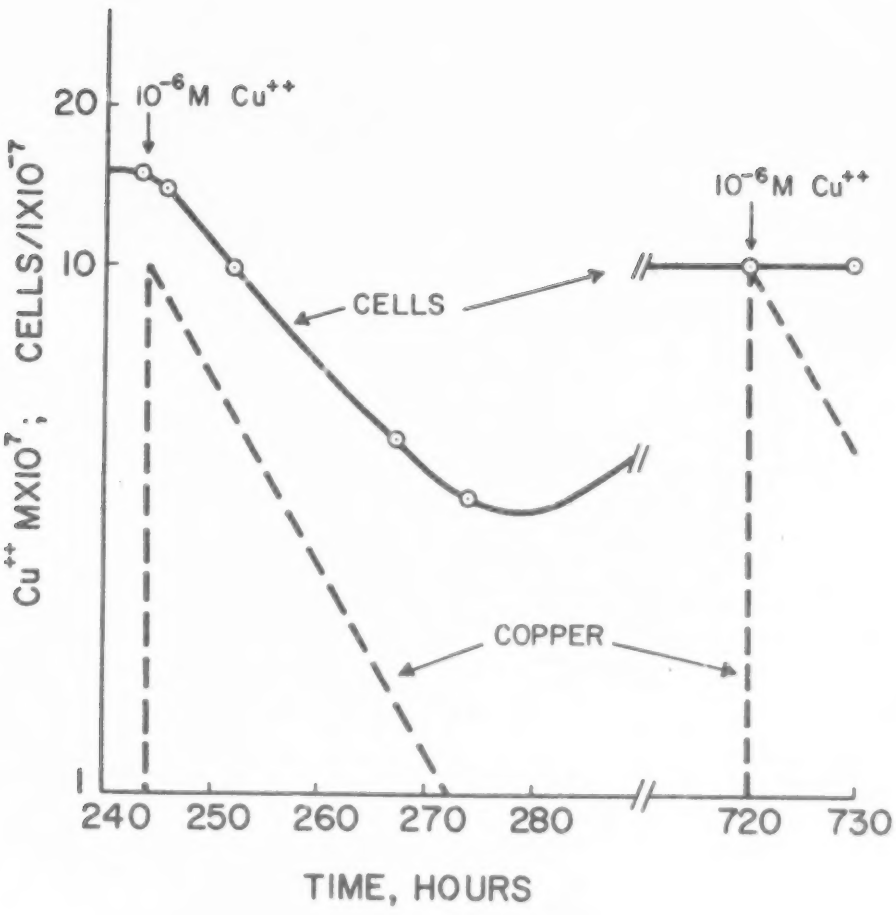

Fig. 5. Effect of first and second addition of 10^{-6} M Cu^{++} added to the reactor of a steady state continuous culture of a marine yeast (pink 1) growth at 0.092/hr.

DISCUSSION

BELSER: Dr. Button, Dr. Jones reported alterations in cellular morphology with the emergence of resistants. Have you observed a similar phenomenon in these kinds of studies?

BUTTON: This is recent work. We have developed a theory of this over the past couple of years and this was experiment number one. If I had had things organized properly, we could have had size distribution profiles throughout this copper perturbation. The only yeast that will work for this has turned out to be tiny and it was below our current facility for measuring size distribution. We have the facility for answering your question but I do not have an answer for your question. I think the probability of what you say, or of what Dr. Jones said, happening, though, is certainly quite great.

JONES: Did the yeast concentrate the metals at all?

BUTTON: The ratio between the copper concentrations and cell mass was 10^6; in other words, if all of the copper had gone into the mass of the cell it would be one part in 10^6 copper. I do not have data, but I cannot see how they could do otherwise. One has on the surface the type of material that has ligands of the type that binds things like heavy metals. As you know, if you do not have a really clean system and you try inoculating, as Dr. Jannasch pointed out, with a small inoculum, it is hard to get things going, but if you use a large inoculum, it is not so hard and this is probably because the cell surfaces are good complexing agents which, of course, has been demonstrated by many people.

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